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Biology of the Sugar-Fermenting Sarcinae

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INTRODUCTION

In 1842, John Goodsir observed a microorganism "of an undescribed form" in the stomach contents of an individual suffering from an affliction of the digestive tract (35). He described the organism as having "the appearance of a wool-pack, or of a soft bundle bound with cord" and, accordingly, proposed the generic term *Sarcina* (Latin for package, bundle) to designate it. In consideration of its occurrence in the stomach, he named it *S. ventriculi* (*ventriculi*, Latin, of the stomach). Goodsir (35), and later Suringar (83), found that the bundles were composed of cells shaped like rounded rectangular parallelepipeds or somewhat flattened spheres. In addition, Suringar (83) established that the organism did not form plates consisting of a single layer of cells, as some authors had come to believe, but that the bundles or packets were approximately cubical. He concluded that the cuboid shape was a result of the organism's characteristic cell division which occurred "alternativement dans les trois directions de l'espace" (83). Neither Goodsir nor Suringar cultivated *S. ventriculi*, but both conducted their morphological studies on specimens obtained from the stomach. Cultivation of the organism, first from garden soil (11, 12) and then from stomach contents (13), was achieved by Beijerinck. This author used a procedure involving inoculation of these materials in a sugar-containing, strongly acidified medium which he incubated anaerobically. Under such conditions, *S. ventriculi* grew abundantly, whereas growth of other microorganisms was largely inhibited. Furthermore, Beijerinck established that *S. ventriculi* is an obligate anaerobe.

After the discovery of the first sarcina, numerous other packet-forming cocci, many of them aerobic, were described and cultured. Some of these organisms were isolated from stomach contents and were erroneously thought to be identical with *S. ventriculi* (*see* 78).

In 1888, Lindner (P. Lindner, Ph.D. Dissertation, Berlin, 1888) observed a large sarcina, morphologically similar to *S. ventriculi*, in spontaneously souring flour paste. The organism was named *S. maxima* and was later isolated in pure culture by Smit (78), who found that it was a strict anaerobe and that it fermented carbohydrates mainly to butyrate, acetate, CO_2, and H_2. Its fermentation differed from that of *S. ventriculi*, which formed no butyrate but accumulated ethyl alcohol as a major product.

Smit (78) mentioned another packet former, *S. paludosa*, similar to *S. ventriculi* in appearance, and probably anaerobic. *S. paludosa* was observed in sewage and in the sludge of polluted rivers, but it was not isolated. Thus, its relationship to other anaerobic sarcinae remains uncertain.

This review is concerned with the biology of the sugar-fermenting, strictly anaerobic sarcinae, namely *S. maxima* and *S. ventriculi*. As will be discussed later, these are the only known species appropriately included in the genus *Sarcina* and are clearly distinct, both morphologically and physiologically, from other packet-forming cocci. Furthermore, they possess properties infrequently observed in other bacteria, such as the ability to grow at *p*H values as low as 1, the formation of extremely large packets, a rapid loss of viability in cultures and, presumably, alternate pathways of pyruvate metabolism. Their unusual characteristics have stimulated the interest of investigators for over a century, as attested by the numerous published reports. The early literature was

reviewed extensively by Smit in 1930 (78) and will not be discussed here, except where pertinent to the presentation.

SELECTIVE ISOLATION AND GENERAL PROPERTIES

Although *S. ventriculi* was originally observed in stomach contents, this organism is not found in the healthy human stomach. Only when the normal stomach environment is altered by the development of certain pathological conditions, such as pyloric ulceration and stenosis, may a selective situation be created which favors the growth of *S. ventriculi* (79). Under these or similarly abnormal circumstances, the flow of food to the intestine is retarded, and the stomach functions as a natural enrichment culture for the anaerobic sarcinae. At the strongly acid *p*H of the stomach (the *p*H of gastric juice being approximately 1) and in the presence of carbohydrates and other nutrients contained in the food, *S. ventriculi* thrives and multiplies rapidly, whereas the growth of other microorganisms is inhibited. The sarcina, whose habitat is soil, is probably ingested with soil particles present in the food.

After Goodsir's original finding, *S. ventriculi* was observed by various investigators in the diseased stomach of humans, in the stomach of rabbits and guinea pigs, on the surface of cereal seeds, and in elephant dung (e.g., 7, 13, 40, 47, 63, 79, 83). However, the organism is more readily found in soil, sand, and mud, and it may be isolated from these materials by the procedures of Beijerinck or by similar procedures (11, 20, 64, 78). The technique constitutes a classical example of selective enrichment culture, inasmuch as no other microorganism can successfully compete with *S. ventriculi* at the extremely low *p*H of the medium (*see* below) in the absence of air and under the conditions provided. (*S. maxima* is a rare exception.) The following is a procedure frequently used in my laboratory for the isolation of *S. ventriculi*.

The enrichment medium contains (g/100 ml of tap water): maltose, technical (Pfanstiehl Lab. Inc., Waukegan, Ill.), 2.0; malt extract broth (powder, BBL or Difco), 5.0; peptone (Difco), 0.5. The *p*H of the medium is adjusted to 2.2 ± 0.1 with diluted acid [e.g., 1 volume of H_2SO_4 (specific gravity 1.84) to 9 volumes of H_2O]. The medium is boiled for 2 or 3 min and, while still hot, it is poured into 60-ml glass-stopper bottles. The bottles, completely filled with medium, are cooled to 40 C in a cold-water bath. Garden soil (preferably saturated with water or growth medium to decrease the amount of air introduced in the bottles) is added to form a layer (2 to 4 mm) on the bottom of each bottle. The bottles are stoppered without trapping air bubbles and are incubated at 37 C.

After 16 to 48 hr, successful enrichments exhibit vigorous gas production. Fine gas bubbles originating from the sarcinae in the sediment rise through the medium and form a layer of foam in the upper part of the bottles. Unless gas production is so active that it resuspends part of the sediment, the supernatant liquid is clear and essentially free of microbial growth since the large sarcina packets remain settled on the bottom. The supernatant liquid of enrichments containing a large number of contaminating organisms (frequently rod-shaped bacteria or yeasts) is turbid; the contamination develops as a result of a rise in *p*H, generally due to reactions between the acid in the culture and material present in the soil. When very alkaline soil is used, the initial *p*H of the medium should be lower than 2.2.

Second enrichment cultures, also in bottles, are prepared without delay by using the same procedure, except that 1 to 2 ml of sediment from the first enrichments is used as the inoculum. After 16 to 48 hr of incubation, cells from the second enrichments may be used to inoculate identical third enrichments for the purpose of accomplishing further dilution of contaminating organisms. Serial dilutions of the growth in these cultures are plated to obtain isolated colonies.

Preparation of third enrichments may not be necessary since the sarcinae in the second enrichments are often so numerically predominant over other organisms that it is advantageous to plate serial dilutions directly from the second enrichments. The following medium (MYA) is used for plating (g/100 ml of distilled water): malt extract broth (powder, BBL or Difco), 2; maltose, technical (Pfanstiehl), 2; yeast extract (Difco), 0.1; agar, 2. The *p*H of the medium is adjusted to 6.0 ± 0.2 with 5% (w/v) KOH and the medium is sterilized. Serial dilutions are prepared in tubes of melted medium MYA at 45 C. These are poured in sterile petri dishes and, after solidification, are incubated anaerobically (e.g., on Bray dishes), or 15 ml of medium MYA is poured on the surface of the medium in each plate (double-layer plates) and the cultures are incubated in air.

Colonies of *S. ventriculi* appear after 10 to 32 hr. Cells from the colonies are transferred to tubes containing medium MYA from which the agar has been omitted. The medium in the tubes is heated for 5 to 10 min in a boiling-water bath, then cooled to 40 C before inoculation. Serial

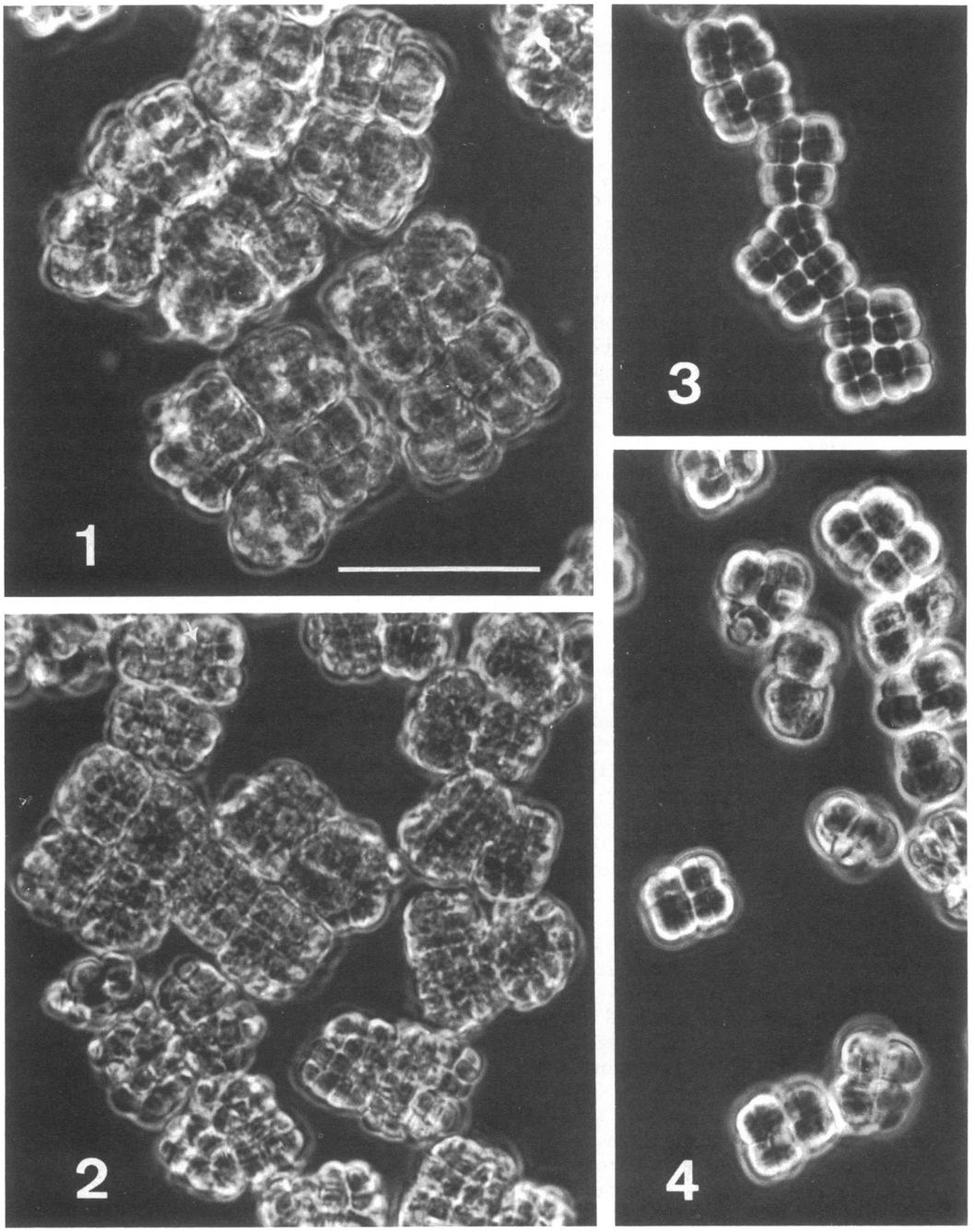


FIG. 1 and 2. *Packets of S. ventriculi strains DP and AL2, respectively. Wet-mount preparations, phase contrast. The photomicrograph of strain DP (Fig. 1) was taken immediately after the organism's isolation from mud; the packets are large and individual cells are not readily apparent. This is typical of packets present in enrichments or in pure cultures soon after their isolation, previous to prolonged subculturing. The marker bar denotes 20 μm.*

FIG. 3 and 4. *Packets of S. maxima strain 11. Wet-mount preparations, phase contrast. The cells in Fig. 3 are regularly arranged in four parallelepiped-shaped aggregates or large packets which include four small eight-celled packets each. Only the top four cells of each small packet are visible. Commonly, S. maxima packets have a more irregular appearance, as in Fig. 4. The magnification is identical with that of Fig. 1 and 2. Figure 3 is from Holt and Canale-Parola (42).*

dilutions of the growth in these tubes are plated as described above to obtain pure cultures.

S. maxima commonly occurs on the hull or outer coat of cereal grains such as wheat, oat, rice, and rye (78). In addition, the organism was isolated from soil and horse manure (47). Crecelius and Rettger (24) reported isolating *S. maxima* from feces of guinea pig. However, since analyses of fermentation products were not carried out, it is not certain that the organism was in fact *S. maxima*. A procedure for the selective isolation of *S. maxima*, described by Smit (78), is summarized below.

The enrichment culture is prepared by adding 50 ml of a 2% aqueous sucrose solution (adjusted to *p*H 2.1 with H_3PO_4) to 2 g of fresh wheat bran in a glass beaker. Hulls and husks of rice or rye may be used instead of wheat bran. The beaker is covered with a watch glass and the culture is incubated at 37 C. After 24 hr the enrichment, when successful, exhibits fermentation with gas production, and large sarcinae as well as other bacteria are present in the sediment. Approximately 1 ml of sediment is transferred to a glass-stopper bottle filled with acidified malt wort (*p*H 1.7 to 2.0). After 24 hr of incubation, the culture is in strong fermentation and emits an odor of butyric acid. A white sediment, consisting largely of sarcinae, is present on the bottom. Further purification is achieved by repeated transfers in similar enrichments. Pure cultures are obtained by plating in malt agar, as described for *S. ventriculi*. Smit (78) reported that these enrichments yielded *S. ventriculi* only exceptionally, and that he never obtained *S. maxima* when he used soil to inoculate this type of enrichment.

Generally, packets of *S. ventriculi* consist of a greater number of cells than those of *S. maxima* (Fig. 1–4). Large packets of *S. ventriculi* (e.g., containing approximately 60 or more cells) tend to assume an irregular or distorted appearance. Many of the cells in these packets have exceedingly flattened shapes and are arranged in a rather disorderly fashion (42, 78). Strains of *S. ventriculi* which consistently form small packets have been isolated (42), and these are especially difficult to distinguish by light microscope examination from *S. maxima*. The packets of the latter organism frequently consist of eight cells, although larger packets are common. Many of the eight-cell packets are arranged in groups of four, forming aggregates which have the shape of a parallelepiped (Fig. 3). Cells of *S. maxima* tend to be more rounded than those of *S. ventriculi*, but they are similarly flattened in the areas of contact with other cells in the packet (Fig. 3, 4).

Early workers reported that cells of *S. ventriculi* are 3 to 4 μm in diameter (35, 78), whereas those of *S. maxima* measure 4 to 4.5 μm (78). However, more recent measurements combining phase-contrast and electron microscope techniques indicated that the diameter of *S. ventriculi* cells is generally close to 2 μm, and that of *S. maxima*, approximately 2.5 μm (18, 42). The discrepancy may be ascribed to strain differences, but it seems more likely that the early light microscope measurements were affected by the difficulty of singling out individual cells in the cubical packets.

Both *S. maxima* and *S. ventriculi* are gram positive, catalase negative, and nonmotile, and they have been reported to form spores, as will be discussed later. *S. ventriculi* grows well between 30 and 40 C, *S. maxima* between 30 and 37 C. In agar media, subsurface colonies of *S. ventriculi* are star-shaped or irregularly cubical, and measure up to several millimeters in diameter. Those of *S. maxima* are smaller, cuboid with protuberances, or unevenly spherical. Surface colonies of both organisms in an anaerobic atmosphere are roundish, often with rugged edges. No pigment is apparent in either sarcina.

PHYSIOLOGY

Nutrition and Growth

S. ventriculi and *S. maxima* have relatively complex nutritional requirements. Growth of both organisms is dependent on the presence in the medium of a fermentable carbohydrate, vitamins, and amino acids, in addition to a supplement of inorganic salts. Canale-Parola and Wolfe (21) reported that a strain of *S. ventriculi*, isolated from Illinois mud, required biotin, nicotinic acid, and 11 amino acids (serine, histidine, isoleucine, leucine, tyrosine, methionine, tryptophan, phenylalanine, arginine, valine, and glutamic acid). The organism grew abundantly in a chemically defined medium containing the above-mentioned vitamins and amino acids, as well as glucose and inorganic salts. Another strain of *S. ventriculi*, isolated in Germany (48), exhibited identical nutritional requirements. Knöll and Horschak (48) studied the nutrition of a strain of *S. maxima* and found that, in addition to the same vitamins and amino acids required by *S. ventriculi*, the organism required thiamine, threonine, alanine, and aspartic acid.

Complex undefined media and cultivation procedures for both sarcinae have been described (18, 20, 21, 54). The latter procedures are similar to those used for other strict anaerobes.

A cultural peculiarity of the anaerobic sarcinae is that the harvesting of mass cultures is greatly facilitated because cells growing in liquid media are present as a sediment on the bottom of the container. Most of the clear supernatant liquid may be siphoned off and the cells may be col-

lected from the remainder of the culture either by centrifugation or by filtration through standard filter paper. The latter method of harvest is used for *S. ventriculi*, since the packets of this organism are retained by filter paper. Under conditions involving slow stirring of actively gassing cultures (the high rate of CO_2 and H_2 evolution providing anaerobiosis), final cell yields of 8 to 12 g (wet weight) per liter were obtained with *S. ventriculi* (18). *S. maxima*, in static cultures, yielded approximately 2.5 g of cells (wet weight) per liter (54). Apparently, this organism prefers a lower redox potential for growth than does *S. ventriculi*; inclusion of sulfhydryl compounds, such as sodium thioglycollate or L-cysteine, in culture media helps initiate its growth. Unbuffered cultures of *S. maxima* or *S. ventriculi* exhibit a *p*H of approximately 4.5 at the end of growth, regardless of the initial *p*H (provided that the latter is 4.5 or higher).

The formation of large packets by *S. ventriculi* poses an intriguing nutritional problem inasmuch as cells located in the interior of the packets are not in direct contact with the growth medium. Either these cells are metabolically inactive, possibly dead, or a mechanism may exist which enables substrates to move across the peripheral layer of cells and reach the inner regions of the packets. As will be discussed later, cells of *S. ventriculi* strains which form large packets are surrounded by a thick, fibrous layer consisting of cellulose or cellulose-like material. This material serves as an intercellular cement that holds the cells together into packets. It may be speculated that diffusion of nutrients from the exterior through this intercellular network of cellulosic material may supply the internal regions of the packets with quantities of substrate sufficient to support a limited level of metabolic activity.

Glucose Fermentation in S. ventriculi

Analyses of *S. ventriculi* fermentation products reported in the early 1930's by Kluyver [Table 1, column 1 (45)] and Smit (78, 79) indicated that growing cells fermented carbohydrates to roughly equimolar amounts of ethyl alcohol and CO_2, and that these compounds accounted for 80 to 90% of the carbon fermented. Only small quantities of H_2 and acetic acid were produced. In accordance with these data, Smit and Kluyver suggested that the pathway of sugar fermentation in *S. ventriculi* was similar to that of the alcoholic fermentation in yeast. However, results of more

TABLE 1. *Glucose fermentation by S. ventriculi and S. maxima*

Product[b]	Amt of products[a]				
	S. ventriculi			*S. maxima*	
	1	2	3	4	5
Carbon dioxide	195	190	190	149	197
Hydrogen	41	170	140	230	223
Formic acid	3	Trace	—	4	NP[e]
Acetic acid	20	90	60	30	40
Butyric acid	—[c]	—	NP[d]	76	77
Lactic acid	—	—	10	21	NP
Succinic acid	—	—	—	5	—
Ethyl alcohol	171	80	100	Trace	NP
Acetoin	4	—	Trace	—	NP
Neutral volatile (as butanol)	—	—	—	—	9
Per cent carbon recovered	99.3	88.3	90.0	100.0	103.5
Oxidation-reduction balance	1.00	1.15	1.12	0.80	0.95

[a] Expressed as micromoles of product per 100 μmoles of glucose fermented.

[b] Fermentation products of growing cells, except for the data in column 2 which were obtained with cell suspensions. Data in column 1 are from Kluyver (45), in column 2 from Milhaud et al. (64), in column 3 from Canale-Parola and Wolfe (20), in column 4 from Smit (78), and in column 5 from Kupfer and Canale-Parola (54). Data in columns 1 and 4 were originally reported as percentages of glucose fermented.

[c] Not reported.

[d] Not present in detectable amounts.

[e] Detected when cells were grown in media containing $CaCO_3$.

recent analyses of products formed by cell suspensions (64) and growing cells (20) cannot be explained entirely on the basis of a yeast-type fermentation (Table 1, columns 2 and 3). Rather, the fermentation products and their ratios resemble those characteristic of the coli-aerogenes group of bacteria.

It has been suggested that a decarboxylase similar to that catalyzing the formation of acetaldehyde and CO_2 from pyruvate in yeast (EC 4.1.1.1), as well as a coli-type pyruvate clastic system, are active in *S. ventriculi* (8). The latter system cleaves pyruvate to acetyl-coenzyme A and formate which, in succeeding steps, is metabolized to H_2 and CO_2 (43, 87, 88, 100). Arbuthnott et al. (2) found that intact cells of *S. ventriculi* incubated at *p*H 7.0 in the presence of pyruvate yielded both CO_2 and H_2 and that the CO_2-to-H_2 ratio was greater than unity. On the other hand, at *p*H 4.9, 1 mole of pyruvate yielded 1 mole each of acetic acid, CO_2, and H_2. These results suggested that, at *p*H 7.0, pyruvate was metabolized by both the decarboxylase and the coli-type clastic system, whereas at the more acid *p*H only the latter system was active. Thus, the proposed occurrence of both systems in *S. ventriculi* may serve to explain the differences between the early fermentation balances (45, 78) and those reported more recently (20, 64; Table 1). Cultural conditions used by the different investigators, or strain peculiarities, may have determined predominance of the activity of one pyruvate metabolizing system over the other.

The evidence supporting the occurrence of two systems for pyruvate metabolism in *S. ventriculi* is preliminary, and it would be desirable to complement it with additional data. Bauchop and Dawes (8) concluded that a yeast-type decarboxylase was active in *S. ventriculi* largely because they found that toluene-treated cell suspensions metabolized pyruvate mainly to acetaldehyde and CO_2, without production of H_2, and that CO_2 evolution from pyruvate was sensitive to the same inhibitors as yeast pyruvate decarboxylase (37, 44). Bauchop and Dawes also reported the presence of the coli-type pyruvate clastic system in *S. ventriculi* (8). They demonstrated the occurrence of both formate dehydrogenase and hydrogenase activities (9), but they did not detect a mechanism yielding formate from pyruvate. However, they inferred that such a mechanism was present in the organism because of the occurrence of the formic hydrogenlyase system (8). Canale-Parola (Ph.D. Dissertation, University of Illinois, Urbana, 1961) reported that cell-free extracts of *S. ventriculi* formed acetyl phosphate from pyruvate in the presence of benzyl viologen or other dyes. He detected phosphotransacetylase

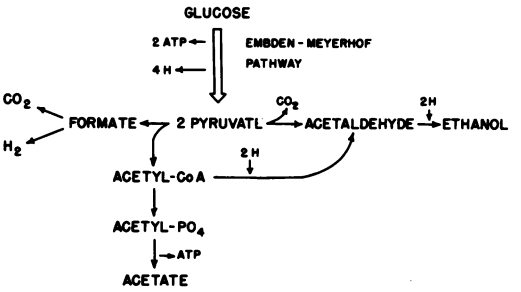


FIG. 5. *S. ventriculi; postulated pathways of glucose fermentation.*

(EC 2.3.1.8) and acetate kinase (EC 2.7.2.1) in the extracts. Valentine and Wolfe (92) also found phosphotransacetylase, but not phosphotransbutyrylase activity, in cell extracts. These data are in agreement with the occurrence of a coli-type pyruvate clastic system in *S. ventriculi* and indicate that the organism produces acetate from pyruvate via the formation of acetyl-coenzyme A and acetyl phosphate.

The presence of both pyruvate metabolizing systems, if confirmed, would suggest that *S. ventriculi* has at least two available paths for the formation of ethyl alcohol from pyruvate (Fig. 5). One, in which coenzyme A does not participate, is identical with that present in yeast and involves the reduction of acetaldehyde formed directly from pyruvate. The other path, similar to that utilized by *Escherichia coli* (25, 26, 72), includes the reduction of acetyl-coenzyme A to acetaldehyde, followed by reduction of this intermediate to ethyl alcohol.

Determinations of radioactivity in products formed by *S. ventriculi* cell suspensions from ^{14}C-glucose labeled in different positions indicated that glucose was metabolized to pyruvate through the Embden-Meyerhof pathway (64). Enzymes associated with this pathway, such as hexokinase (EC 2.7.1.1), fructosediphosphate aldolase (EC 4.1.2.13), and triosephosphate dehydrogenase (EC 1.2.1.12) were detected in cell-free extracts of *S. ventriculi* (2, 22).

Those strains of *S. ventriculi* that were tested did not ferment pentoses (21, 78). Milhaud et al. (64) reported that paper chromatography of *S. ventriculi* cell extracts did not reveal the presence of tricarboxylic acid cycle intermediates.

Synthesis of Cellulose by S. ventriculi

Disagreement developed among the early investigators as to whether *S. ventriculi* was to be considered a "vegetable" organism (*see* 78, 79, 83). Suringar (83) tested whether cellulose, which is commonly found in plant cell walls,

was also present in the walls of *S. ventriculi*. He observed that treatment of the organism with Schultz's reagent for cellulose (an aqueous solution of $ZnCl_2$, KI, and I_2) stained the sarcina walls red to violet, an indication that the polysaccharide was present. Smit (78, 79) confirmed Suringar's observations and, furthermore, found that *S. maxima* and *S. methanica* gave a negative cellulose stain reaction.

A material possessing the properties of cellulose was purified by Canale-Parola et al. (18) from a strain of *S. ventriculi* which stained red to violet with Schultz's reagent. This material, which accounted for up to 19% of the total dry weight of the cells (22), was identified as cellulose inasmuch as it stained strongly with the cellulose stain, exhibited the solubility behavior of this polysaccharide, and, upon hydrolysis, yielded only glucose. However, it is not excluded that in the organism the polysaccharide may have a slightly different structure which is altered during the purification procedure. For example, it may be present as a derivative of cellulose, with groups bound to the glucosyl units.

Delaporte (27) reported that a strain of *S. ventriculi* that she tested did not exhibit the color reaction for cellulose and that examination of the cells by infrared spectroscopy gave no evidence for this polysaccharide. This report is not surprising since strains of *S. ventriculi* containing either relatively small quantities of cellulose or no detectable cellulose have been observed (18, 42).

The metabolic steps leading to cellulose synthesis in *Acetobacter xylinum* and *A. acetigenum* have been studied by several investigators, as reviewed by Asai (3). Enzymes involved in the synthesis of cellulose by these bacteria (31, 34, 38) were detected in *S. ventriculi* by Canale-Parola and Wolfe (22). These investigators found hexokinase, phosphoglucomutase (EC 2.7.5.1), and uridine diphosphate glucose pyrophosphorylase (EC 2.7.7.9) activities in cell extracts of the organism.

Carbohydrate Metabolism in S. maxima

Carbohydrate fermentation by *S. maxima* yields products (Table 1) similar to those formed by clostridia exhibiting a butyric type fermentation (29, 84, 100). Kupfer and Canale-Parola (55) determined the radioactivity in products accumulated by cell suspensions of *S. maxima* incubated with glucose-*1*-^{14}C or glucose-*6*-^{14}C. The results showed that the CO_2 formed was derived neither from carbon 1 nor carbon 6 of glucose, and the labeling patterns of acetate and butyrate indicated the presence of the Embden-Meyerhof pathway. Furthermore, enzymes of this pathway (fructosediphosphate aldolase and triosephosphate dehydrogenase) were detected in cell-free extracts of *S. maxima*.

Cleavage and oxidation of pyruvate by clostridia yields acetyl-coenzyme A (or acetyl phosphate), CO_2, and H_2 (53, 100). As previously mentioned, coliform bacteria (*Enterobacteriaceae*) catabolize pyruvate anaerobically with the formation of acetyl-coenzyme A (or acetyl phosphate) and formate. These two types of pyruvate cleaving systems are sometimes referred to as "phosphoroclastic" reactions. Cell-free extracts of bacteria which possess a clostridial phosphoroclastic reaction catalyze a rapid exchange between CO_2 and the carboxyl group of pyruvate (1, 68, 82, 95, 96, 98), whereas formate-pyruvate exchange is catalyzed by extracts of bacteria utilizing the phosphoroclastic reaction typical of *E. coli* (81, 89, 90, 99). Experiments involving exchange between $^{14}CO_2$ or H^{14}-COOH and pyruvate were conducted by Kupfer and Canale-Parola (54) to investigate the mechanism of pyruvate cleavage in *S. maxima*. It was found that extracts prepared from cells in the stationary phase of growth (having no detectable formic dehydrogenase activity) catalyzed approximately 100% of theoretical exchange between $^{14}CO_2$ and pyruvate, whereas no exchange between H^{14}-COOH and pyruvate was detected. These results were confirmed by other investigators (R. B. Hespell and R. P. Mortlock, *personal communication*), who found that *S. maxima*, like other bacteria which utilize a clostridial type of pyruvate cleavage, requires catalytic levels of coenzyme A for the CO_2-pyruvate exchange portion of the phosphoroclastic reaction.

Kupfer and Canale-Parola (54) reported that cell-free extracts of *S. maxima* which were incubated with pyruvate in the presence of methyl viologen formed acetyl phosphate, CO_2, and H_2. Phosphotransacetylase and acetate kinase activities were detected in the extracts, indicating that, as in *S. ventriculi*, acetate is produced via acetyl-coenzyme A and acetyl phosphate (54, 55). Experiments with labeled glucose showed that *S. maxima* formed butyrate from two-carbon fragments (acetyl portion of acetyl-coenzyme A) resulting from the decarboxylation of pyruvate (55). Butyryl-coenzyme A dehydrogenase (EC 1.3.99.2), phosphotransbutyrylase, and butyrate kinase (EC 2.7.2.a) were present in the extracts. It was concluded that *S. maxima* produces butyrate from acetyl-coenzyme A by mechanisms similar to those occurring in saccharolytic clostridia.

The nonheme, iron protein ferredoxin (59, 65, 91) was purified from extracts of *S. maxima* by

Kupfer and Canale-Parola (54). Sarcina ferredoxin replaced *Clostridium pasteurianum* ferredoxin in the phosphoroclastic system of the latter organism and accepted electrons from pyruvate in the presence of *S. maxima* extracts. Since, in clostridia, ferredoxin serves as an electron carrier between pyruvic dehydrogenase and the hydrogenase system (59, 65, 91), it was expected that this iron protein would play a similar role in *S. maxima*. A hydrogenase which accepted electrons from reduced methyl viologen was detected in extracts of *S. maxima* (54). However, in the presence of the same extracts, reduced ferredoxin from *S. maxima* or *C. pasteurianum* did not transfer electrons to the hydrogenase. One interpretation of these results is that, in reaction mixtures, the hydrogenase system of *S. maxima* is partially labile, so that it no longer accepts electrons from the natural carrier ferredoxin, whereas electrons are transferred to it from the less-specific methyl viologen. A second interpretation is that ferredoxin, in *S. maxima*, does not participate in the reactions involved in hydrogen production from pyruvate.

A role for ferredoxin in *S. maxima* was uncovered by experiments conducted to determine whether this protein transferred electrons to pyridine nucleotides (54). *S. maxima* extracts, treated with diethylaminoethyl cellulose to remove ferredoxin, were incubated in the presence of pyruvate and nicotinamide adenine dinucleotide phosphate (NADP). Pyridine nucleotide reduction was observed only upon addition of ferredoxin to the mixture, indicating that NADP reduction from pyruvate in *S. maxima* is ferredoxin dependent. Participation in the generation of reduced NADP for biosynthetic reactions may constitute an important function of ferredoxin in *S. maxima*.

Kupfer and Canale-Parola (54) found that resting cells and cell extracts of *S. maxima* produced CO_2 and H_2 from formate as well as from pyruvate. Whole cells evolved equimolar amounts of CO_2 and H_2 from formate. These observations indicate that CO_2 and H_2 are formed by this organism through the mediation of a hydrogenlyase system and that formate may be an intermediate in the production of these gases from pyruvate.

A formate-pyruvate exchange system could not be demonstrated in *S. maxima*, probably because of its lability or inactivity under the conditions used (54). However, as in *S. ventriculi*, the presence of a coli-type phosphoroclastic reaction may be inferred in view of the occurrence of the hydrogenlyase system. The accumulation in the medium of small amounts of formate by cells of *S. maxima* fermenting glucose (Table 1) is an indication that formate is an intermediate of glucose metabolism by this bacterium.

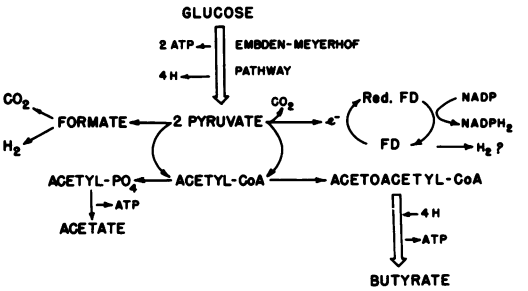


FIG. 6. *S. maxima; postulated pathways of glucose metabolism.*

Apparently, *S. maxima*, like *S. ventriculi*, possesses at least two distinct pathways of pyruvate metabolism (Fig. 6). Although the evolutionary significance of the double pathway is uncertain at present, it is probable that its presence confers considerable metabolic versatility to these organisms.

Growth at Extremely Acid pH

An interesting aspect of the physiology of *S. maxima* and *S. ventriculi* is their ability to grow within an extraordinarily wide *p*H range. Smit (78, 79) reported growing *S. ventriculi* at *p*H values slightly lower than 1, as well as in media with an initial *p*H of 9.8. *S. maxima* grows within a similar *p*H range (78).

After prolonged subculturing in media with an initial *p*H close to neutrality, *S. ventriculi* grows poorly (or not at all) when transferred into an acid medium (*p*H 4.5 or lower). However, strains freshly isolated from nature (by using enrichments at *p*H of approximately 2) can be subcultured indefinitely in highly acid media. The growth rate and yields of freshly isolated *S. ventriculi* do not vary appreciably whether the organism is grown at *p*H 3.5 or in media adjusted to an initial *p*H of 7.0 (Canale-Parola, *unpublished data*).

The considerable acid tolerance of the anaerobic sarcinae is equalled by few known bacteria, i.e. *Thiobacillus thiooxidans* (77). As pointed out by Doetsch et al. (28), "no adequate explanation for this remarkable property has been formulated. Those explanations which have been offered, such as Umbreit's (86) "submarine theory," usually invoke a barrier, i.e., the cytoplasmic membrane, which prevents hydrogen ions from gaining access to the interior of the

cell." It is generally believed that the overall internal *p*H of bacteria is approximately neutral (80). However, few investigations on the intracellular hydrogen ion concentration of microorganisms have been reported (17, 23, 39), and it has not been established whether intraprotoplasmic gradients or compartmentation of hydrogen ion concentrations exist in the procaryotic cell (although their existence is to be suspected).

When *S. ventriculi* and *S. maxima* grow in extremely acid environments, at least some of the cell structures are exposed to the high external concentration of hydrogen ions. One of these structures is the cytoplasmic membrane, or its outer layer. Enzymes associated with the cytoplasmic membrane (57, 71, 73), as well as extracellular enzymes, presumably remain active at the extremely acid *p*H. Furthermore, the exceptional acid tolerance of the anaerobic sarcinae (and *T. thiooxidans*) may reflect the existence of structural and biochemical differences between the membranes of these organisms and those of less acid tolerant bacteria.

Rapid Loss of Viability and Spore Formation

Cultures of *S. maxima* and *S. ventriculi* in liquid media are no longer viable after 2 to 4 days of incubation. This rapid loss of viability has puzzled and inconvenienced investigators, who have transferred the organisms daily or on alternate days to maintain viable cultures (20, 64, 78). Apparently, a metabolic product (or products) toxic to the cells is involved in the rapid death. This is suggested by the observation that cultures of *S. ventriculi* remain viable for 7 days when the organism is cultivated under growth-limiting conditions [in liquid media containing low concentrations of carbohydrate or yeast extract (20)]. Furthermore, both *S. ventriculi* and *S. maxima* can be maintained for up to 2 months when inoculated in a depression or well melted through the surface of a relatively large amount of agar medium contained in a flask. These "depression" stock cultures are kept at 30 or 37 C to allow continuous growth of the cells; growth is confined to the depression, and any toxic product formed becomes diluted throughout the large amount of agar medium (20).

It is possible that the toxic effect results, at least in part, from the combined action of accumulated products (e.g., fatty acids) and of the acid *p*H present in cultures after prolonged fermentation (approximately 4.5 for both sarcinae). At acid *p*H values, acetic and butyric acids are present to a large extent in the undissociated form, and they penetrate cells more readily than in neutral or alkaline environments (80). The toxicity of undissociated acids for bacteria has been documented, although the mechanisms involved have not been elucidated (30, 56). The anaerobic sarcinae may be more sensitive than other bacteria to the lethal effects of the undissociated acids.

Lyophilized cells of *S. ventriculi* and *S. maxima* generally do not survive longer than 1 month. A more successful procedure routinely used in my laboratory for the maintenance of these organisms was adapted from a method described by Silver et al. (76) for mammalian cells. The sarcinae are suspended in sterile growth medium to which either glycerol (0.1 ml per ml of medium) or dimethyl sulfoxide (0.04 ml per ml of medium) is added. Approximately 1-ml samples of the suspension are sealed in small glass vials, which are then cooled to −65 C in an ethyl alcohol bath. (The approximate rate of cooling is one degree per minute.) The vials are finally transferred to a liquid nitrogen storage container (−196 C). The cells in these preparations remain viable for at least 6 months.

If cells from pure cultures of anaerobic sarcinae are mixed with sterilized soil, sand, or chalk, they do not survive for longer than 2 or 3 days (78, 79; Canale-Parola, *unpublished data*). This observation is especially intriguing since both *S. ventriculi* and *S. maxima* are widespread in nature and can be cultivated from soil and other materials which have been stored for several years. Smit explored the possibility that the anaerobic sarcinae remain viable for long periods of time in nature because of spore formation (78, 79). He never observed spores in cultures, nor did he have success in inducing sporulation. Experiments in which he tested the heat resistance of *S. ventriculi* and *S. maxima* naturally present in sand and bran indicated that the anaerobic sarcinae could no longer be cultured from sand heated for 10 min at 75 C or from bran maintained at 60 C for 15 min. Smit concluded that spore formation by *S. ventriculi* and *S. maxima* is highly improbable, and he proposed that these organisms "exist in natural materials like soil and sand and retain their vitality, in a form until now unseen and unknown, being different from the known sarcina-packets and capable of surviving for a prolonged period" (79). According to Smit, the "natural," stable sarcina form—of undetermined morphology—is somewhat more heat resistant than sarcina packets; i.e., packets of *S. ventriculi* mixed with sterilized sand are killed in 10 min at 65 C, whereas the stable form requires 10 min at 75 C.

He suggested that the more resistant form, under appropriate conditions, gives rise to sarcina packets, but that this process is irreversible because the packets cannot generate the stable form (79).

More recently, Knöll reported that he succeeded in inducing sporulation of the anaerobic sarcinae. His report included time-lapse photomicrographs illustrating the development of *S. ventriculi* and *S. maxima* packets from spores (47). Spores were obtained by a procedure involving incubation of growing cells in a CO_2 atmosphere, followed by addition of phosphate buffer and alkali which rapidly raised the *p*H of the cultures to 7.5 for *S. ventriculi* and to 9 to 10 for *S. maxima*. Endospores developed during further incubation of the cultures in nitrogen gas. *S. ventriculi* formed spherical spores, whereas spores of *S. maxima* were oval. The spores were refractile and heat resistant (no thermal resistance data reported), and were colored green by Wirtz's (97) spore stain (47; Knöll, *personal communication*).

The ability to form spores would provide an explanation for the ubiquitous occurrence and long survival of the anaerobic sarcinae in natural environments. On the other hand, it is difficult to reconcile the presence of sarcina spores in nature with Smit's observation that the sarcinae cannot be cultured from pasteurized natural materials. Experiments similar to those reported by Smit were conducted in my laboratory. Portions of soil samples, which had been stored for 2 to 3 years, were heat treated (2 or 3 min at 100 C). Anaerobic sarcinae never developed in enrichment cultures inoculated with the heat-treated soil, whereas they developed in identical cultures inoculated with the nonheated portions of the same samples. Nutrient-agar plates streaked with the heat-treated material invariably yielded colonies of aerobic sporeforming rods, indicating that the conditions used were not lethal to bacterial spores. These results and those of Smit's experiments suggest that the long survival of the anaerobic sarcinae in natural environments is not necessarily due to their ability to form heat-resistant spores.

FINE STRUCTURE

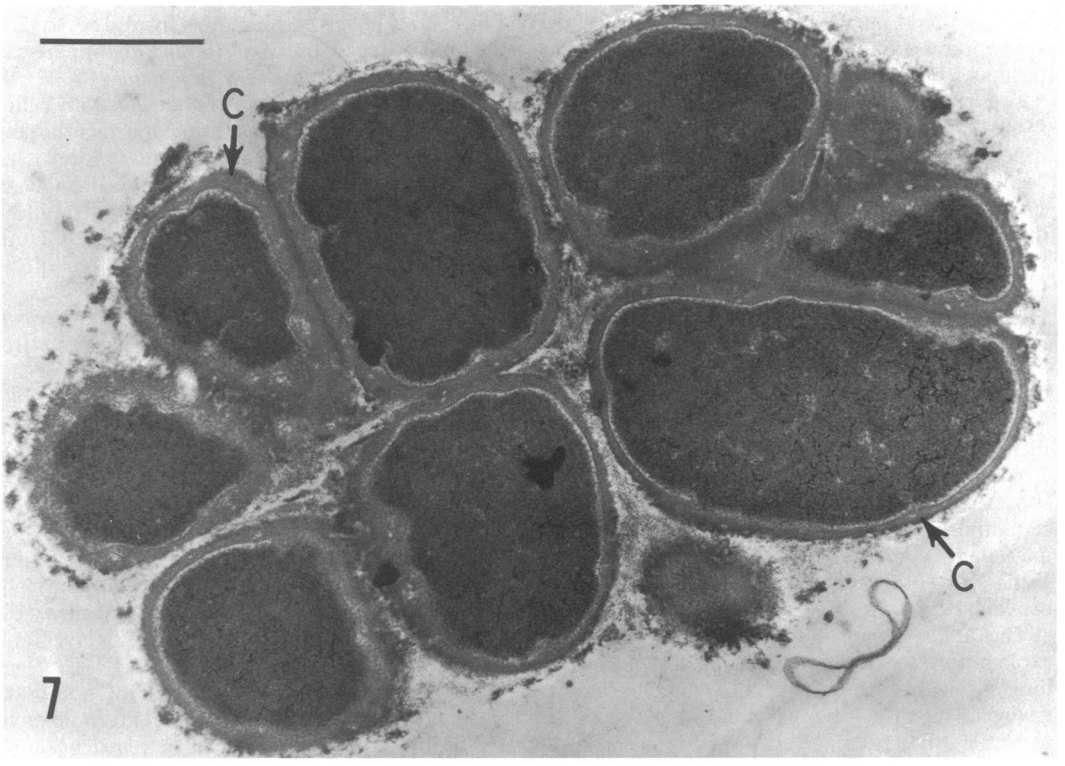
The overall structural organization of the anaerobic sarcinae is that typical of procaryotic cells. However, *S. maxima* and *S. ventriculi* differ from one another with respect to certain structural features. The most obvious of these is a thick, fibrous layer which is generally present on the outer surface of the wall of *S. ventriculi* (Fig. 7–9), but is not observed in *S. maxima* (18, 42; Fig. 10). The layer measures from 150 to 200 nm in thickness, but it is thinner or apparently absent in some strains of *S. ventriculi* (18, 42). It was found that treatment of the cells with cellulose solvents or cellulase preparations largely destroyed this layer. These observations, as well as previously mentioned chemical analyses of material removed from the cells by cellulose solvents, indicated that the thick layer is composed either totally or in great part of cellulose, or of a closely related compound (18).

Each cell within a given packet of *S. ventriculi* is enveloped by the cellulose layer, which frequently appears to be continuous with or attached to the layer surrounding adjacent cells (18, 42). Possibly the polysaccharide layer serves as a matrix or a cementing substance which binds cells of *S. ventriculi* into the large, many-celled packets characteristic of this organism. This interpretation is in accord with reports that strains of *S. ventriculi* which deposit little or no cellulose tend to form packets consisting of relatively few cells, loosely attached to one another (18, 42). A nutritional role for the cellulose layer was suggested earlier in this review.

The appearance of the cell wall of both sarcinae is similar to that of other gram-positive walls (Fig. 8, 10). The wall of *S. ventriculi* is 30 to 40 nm thick, that of *S. maxima* approximately 40 nm (18, 42, 49).

Knöll and Niklowitz (49) examined thin sections of *S. ventriculi* by electron microscopy and found spherical-to-ovoid inclusions, approximately 250 nm in diameter, in the cytoplasm of this organism. According to these authors, the inclusions were separated from the surrounding cytoplasm by an osmiophilic layer and consisted of fine granules which, in some cases, appeared to be arranged in lamellar structures. Knöll and Niklowitz reported that these inclusions occurred in all phases of cellular growth, near the periphery of the cell. Morphologically similar inclusions were observed in thin sections of *S. ventriculi* by Holt and Canale-Parola (42). However, these investigators did not detect either a limiting layer or lamellar structures associated with the inclusions, and suggested that the latter may represent sites of polysaccharide storage. Generally these or similar granules were not observed in the cytoplasm of *S. maxima* (42).

The nuclear material of both sarcinae, as viewed in thin sections by electron microscopy, frequently appears to be dispersed throughout the cytoplasm (42, 49). Delaporte (27), who used light microscopy to investigate the nuclear structure of *S. ventriculi*, reported a similar type of organization. She found that the nucleus of this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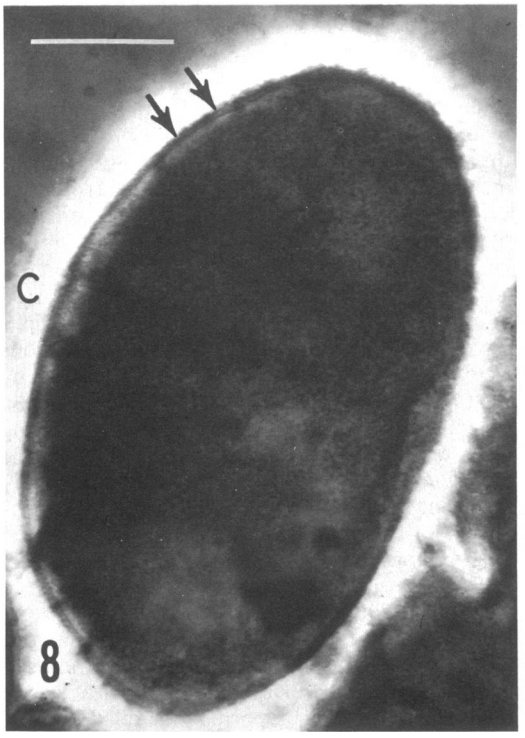
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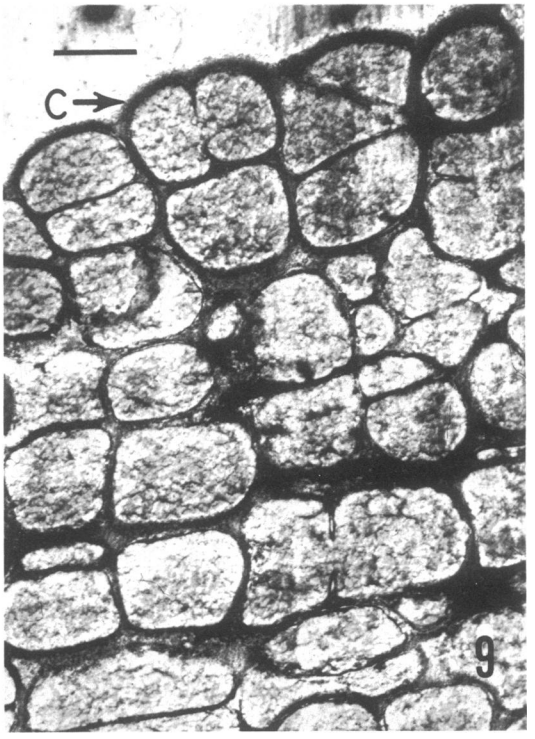
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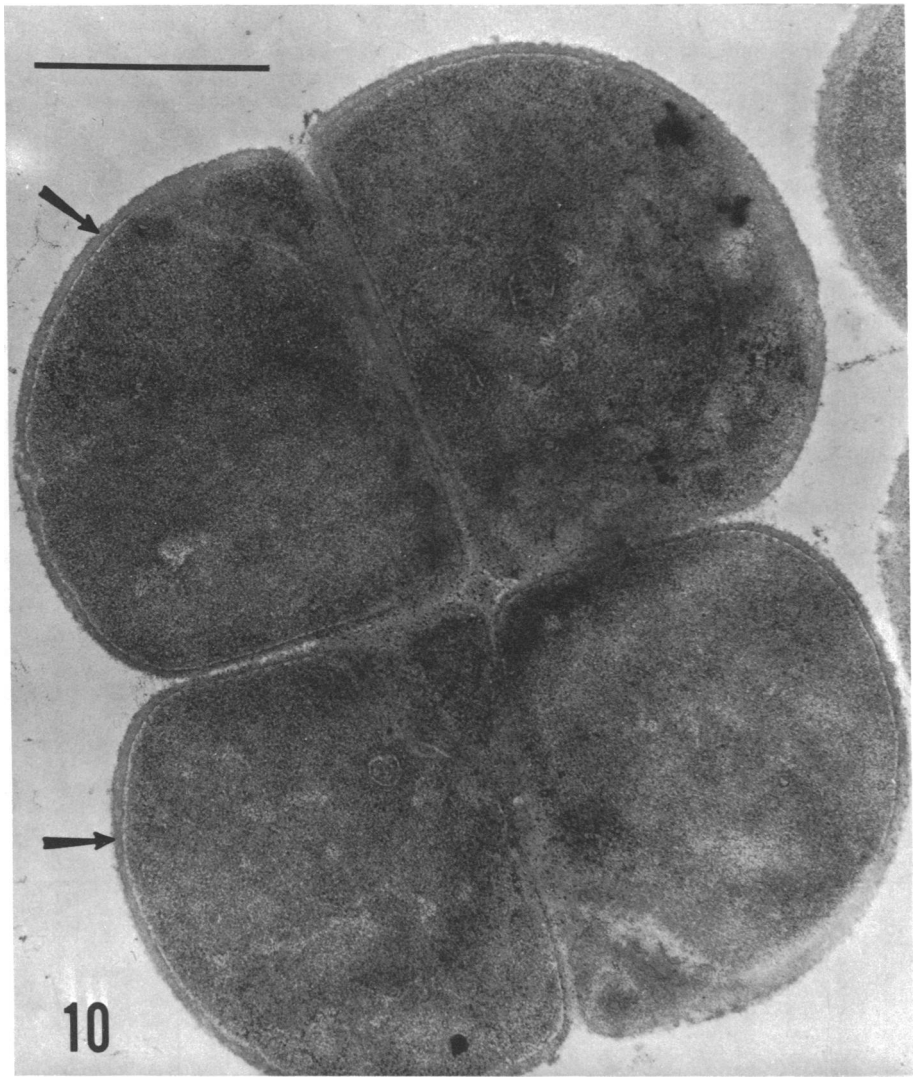

FIG. 10. *Electron micrograph of a thin section of S. maxima strain 11. The arrows point to the cell wall. Embedded in Epon 812, stained with lead hydroxide (42). The marker bar denotes 1 μm. From Holt and Canale-Parola (42).*

FIG. 7. *Electron micrograph of a thin section of S. ventriculi strain AL. The cells are surrounded by a thick cellulose layer (C) which, in this preparation, obscures the cell wall. The embedding material was the epoxy resin Epon 812. Stained with lead hydroxide (42). The marker bar denotes 1 μm. From Holt and Canale-Parola (42).*

FIG. 8. *Electron micrograph of a section through a S. ventriculi cell located at the outer edge of a packet. In this unstained preparation, the cell wall (arrows) is visible and the cellulose layer (C) appears as a clear area surrounding the cell. Strain EC-1. The embedding material was Vestopal (18). The marker bar denotes 0.5 μm. From Canale-Parola et al. (18).*

FIG. 9. *Electron micrograph of a section through a large packet of S. ventriculi strain EC-1. Prepared by the same procedure as the section in Fig. 8, but stained with 1% (w/v) lead acetate. The cellulose layer (C) appears as a dark material surrounding each cell. Note the flattened shape of many of the cells. The marker bar denotes 1 μm. From Canale-Parola et al. (19).*

organism is fragmented into a large number of chromatin granules connected with each other by chromatin threads, an arrangement which allows a large contact area between the nuclear material and the cytoplasm. Generally, this chromatin network is at the periphery of the cytoplasm. A similar organization of the nuclear material has been observed in a variety of bacteria, such as *Azotobacter* species and some of the coliforms (66). Under certain conditions (e.g., in the presence of air), the nuclear granules of *S. ventriculi* merge and the nucleus may contract into a dense, central mass of homogeneous appearance. Delaporte (27) observed that, during division, the nuclear material of *S. ventriculi* moves away from the region where the transverse cell wall is being formed. A bridge of nuclear material connects the two halves of the nucleus and persists until the transverse cell wall is completed. Knöll and Niklowitz (49) reported that, in thin sections of *S. ventriculi*, the nucleus exhibits different shapes according to the stage of cell division and has the appearance of a spongy, coarsely reticulated material made up of thready elements measuring 3.5 nm in diameter.

TAXONOMY OF PACKET-FORMING COCCI

In the years following Goodsir's discovery of *S. ventriculi*, other investigators described and cultivated a variety of packet-forming cocci, many of which were strict aerobes (*see* 78, 79). In time, essentially all of the packet-formers came to be grouped in the genus *Sarcina*, family *Micrococcaceae*. (One exception is *Thiosarcina rosea*.) *S. ventriculi*, the first sarcina to be described, was designated the type species of the genus (16).

The inclusion of the packet-forming cocci into a heterogeneous taxonomic assemblage encountered some opposition, as pointed out by van Niel (93):

> "It would not be surprising to find that bacteriologists familiar with these organisms balk at the notion that the aerobic *S. lutea*, the anaerobic *S. ventriculi*, *S. maxima*, and *S. methanica*, exhibiting an alcoholic, butyric acid, and methane fermentation, respectively, the halophilic *S. gigantea*, and the motile, sporeforming *S. ureae* represent a group of phylogenetically closely related types."

There is no reason to believe that the ability of packet formers to divide in three planes perpendicular to one another reflects a natural relationship closer than that existing among organisms dividing in one or two perpendicular planes, or in random planes. And, in fact, extensive experimental evidence has clearly indicated that extreme diversity is present among the packet-forming cocci.

Barker's studies on the methane bacteria dem-

TABLE 2. *Guanine plus cytosine (GC) content of the DNA of packet-forming cocci*

No.	Organism	Strain[a]	Reference	Method[b]	GC (moles %)
1	*Sarcina lutea*	ATCC 272	14	1	73.7
2	*S. lutea*	ATCC 381	19	2	73.5
3	*S. lutea*	16	69	1	73.3
4	*S. lutea*	ATCC 382	14	1	72.3
5	*S. lutea*	SG 140	94	4	70.7
6	*S. lutea*	17	69	1	67.5
7	*Sarcina flava*	10	69	1	73.3
8	*S. aurantiaca*	ATCC 146	69	1	68.0
9	*Sporosarcina (Sarcina) ureae*	CCM 858	15	3	44.0
10	*S. ureae*	ATCC 6473	4	1	43.0
11	*S. ureae*	CCM 1743	15	1	41.5
12	*S. ureae*	CCM 204	15	1	40.7
13	*S. ureae*	CCM 871	15	1	40.0
14	*S. ureae*	CCM 380	15	3	39.2
15	*Sarcina ventriculi*	AL	19	2	30.6
16	*S. ventriculi*	KVI/VIG	74	4	30.5
17	*S. maxima*	11	19	2	28.6
18	*S. maxima*	48	74	1	29.0

[a] Abbreviations: ATCC, American Type Culture Collection; CCM Czechoslovak Collection of Microorganisms.

[b] Method used to determine the GC content of the DNA: 1, thermal denaturation (61); 2, buoyant density in CsCl (60, 75); 3, ratio $E_{260} : E_{230}$ at *p*H 3 (32); 4, hydrolysis and chromatography (94).

onstrated that these organisms constitute a distinct physiological group (6). He proposed that they be included in a family (*Methanobacteriaceae*) comprising genera recognizable on the basis of morphological features. Within this scheme, he grouped the packet-forming methanogenic cocci in the genus *Methanosarcina*, which he subdivided into two species, *M. barkerii* and *M. methanica*.

Much attention has been focused on the taxonomic status of the flagellated, sporeforming *S. ureae*. It was found that this bacterium forms true endospores, inasmuch as they are heat resistant (10, 33, 51, 58), contain dipicolinic acid (85), and are ultrastructurally similar to spores of *Bacillus* species (62). Furthermore, the guanine + cytosine (GC) content in the deoxyribonucleic acid (DNA) of *S. ureae* differs greatly from that of aerobic, nonsporeforming sarcinae (Table 2). These findings, as well as other morphological and physiological considerations, convinced numerous investigators that *S. ureae* should be removed from the genus *Sarcina* and placed in a separate genus (15, 51, 58) named *Sporosarcina*, as previously proposed by Orla-Jensen (67) and Kluyver and van Niel (46). Some authors believe that, because of biochemical and morphological similarities with species of *Bacillus*, the genus *Sporosarcina* should be included in the family *Bacillaceae* (51, 58). However, recent DNA-ribonucleic acid (RNA) and DNA-DNA hybridization studies (41) indicated that, although there may be a genetic relationship between *S. ureae* and species of *Bacillus*, this relationship is restricted to a very small portion of the total genome. As pointed out by Herndon and Bott (41), it may be desirable to postpone the decision to include the genus *Sporosarcina* in the family *Bacillaceae* until additional information is available on the genetic homology between representatives of these two taxa.

Aerobic, nonsporeforming, nonmotile, packet-forming cocci constitute a group containing approximately 68 to 74 moles per cent GC in their DNA (Table 2). These organisms are similar to or indistinguishable from species of *Micrococcus* both physiologically (5) and with respect to the GC content of their DNA (14, 69, 70). Kocur and Martinec (50, 52) and Baird-Parker (5) found that the formation of packets by aerobic cocci is an unstable property. Many strains, originally designated as species of *Sarcina*, when cultured under certain conditions, lost the ability to form packets. These authors concluded that aerobic, nonsporeforming, nonmotile cocci hitherto classified as species of *Sarcina* should be assigned to the genus *Micrococcus*. Kocur and Martinec (52) suggested that the genus *Sarcina* should include only anaerobic species, that is, the methane sarcinae as well as *S. maxima* and *S. ventriculi*.

Determinations of the GC content in the DNA of *S. maxima* and *S. ventriculi* (19, 74) indicated that these organisms are phylogenetically remote from aerobic strains designated as species of *Sarcina* (Table 2). Moreover, cells of anaerobic sarcinae generally maintain their characteristic arrangement in packets under a variety of cultural conditions (19). On the basis of these findings, and in consideration of Barker's proposal to assign the methanogenic bacteria to a separate family (6), Canale-Parola et al. (19) concluded that only the anaerobic, sugar-fermenting species (*S. ventriculi* and *S. maxima*) should be retained in the-genus *Sarcina*.

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